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Award Number: W81XWH-08-2-0042

TITLE: Catalytic bioscavengers human butyrylcholinesterase and paraoxonase sequestered to the center for CNS

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REPORT DATE: April 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

XXX Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
30-04-2009	Annual	01 APR 2008 - 30 MAR 2009

4. TITLE AND SUBTITLE
Catalytic bioscavengers human butyrylcholinesterase and Paraoxonase Sequestered to the Center for CNS

5a. CONTRACT NUMBER
W81XWH-08-2-0042

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Ruben J. Boado, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
Institute Chemical Defense
Aberdeen Proving Ground, MD
21010-5400

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Background: Chemical nerve gas agents cause mortality via action within the central nervous system (CNS). In addition, sub-lethal exposure to nerve gas agents causes chronic neuropathology. Chemical nerve gas agents are small lipid-soluble molecules that rapidly cross the blood-brain barrier (BBB) and enter brain from blood. Therefore, it is important that new treatments for chemical nerve gas exposure be engineered to also cross the human BBB. Organophosphate chemical nerve gas agents are rapidly inactivated by serum enzymes such as paraoxonase (PON)-1 and butyrlcholinesterase (BCE). However, these enzymes do not cross the BBB.

Objective/Hypothesis: The endogenous human insulin receptor (HIR) on the BBB is a transport system not only for circulating insulin, but also for certain peptidomimetic monoclonal antibodies (MAb) that undergo receptor-mediated transport across the BBB via the endogenous HIR. The HIRMAb acts as a molecular Trojan horse, and can ferry into brain a fused protein, such as PON-1 or BCE. Once across the BBB, the HIRMAb-enzyme fusion protein may inactivate nerve gas within the CNS.

Study Design: A chimeric HIRMAb has been genetically engineered that is active at the human BBB, and rapidly crosses the Rhesus monkey BBB in vivo. A genetically engineered fusion protein of the chimeric HIRMAb and human PON-1 or human BCE will be evaluated. These fusion proteins are transiently expressed in COS cells following the dual transfection with the heavy chain gene and the light chain gene expression plasmid DNA. The relative potency of these fusion proteins as organophosphate inactivators will be measured following the expression and affinity purification of the fusion protein. The fusion protein will be radiolabeled and injected intravenously into an adult Rhesus monkey for determination of plasma pharmacokinetics and in vivo BBB transport.

Relevance: Fusion proteins that are comprised of organophosphate inactivating enzymes that can cross the BBB provide a double layer of protection against chemical nerve gas agents, in that these fusion proteins degrade nerve gas both in the blood and in the brain behind the BBB. Transportability across the BBB is important, because chemical nerve gas agents cause death by action in the CNS following transport across the BBB. The fusion proteins may be mass produced and formulated as stable products that can be stored and deployed in the field for protection against acute chemical nerve gas attacks.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Dr. Ruben J. Boado
Unclassified	Unclassified	Unclassified		6	19b. TELEPHONE NUMBER *(include area code)* 310-917-1275

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

Introduction

The purpose of this research is to genetically engineer, express, and validate 2 novel biopharmaceutical fusion proteins, designated AGT-185 and AGT-186. Both proteins are fusion proteins of human paraoxonase (PON)-1 variants and a genetically engineered monoclonal antibody (MAb) against the human insulin receptor (HIR). The 2 proteins are intended for development as new treatments of the brain in organophosphate chemical nerve gas attacks.

Chemical nerve gas agents are organophosphates (OP), and rapidly degraded by enzymes with organophosphatase activity. Human PON-1 is the most potent human protein with organophosphatase activity, and is being considered as a new treatment of OP toxicity. However, OP agents kill via a mechanism of action within the central nervous system (CNS). Therefore, it is important that new agents against OP toxicity be developed that are active within the brain. PON-1, like other large molecule biopharmaceuticals, does not enter the brain, because brain penetration is blocked by the blood-brain barrier (BBB).

Recombinant proteins such as PON1 can be re-engineered for transport across the human BBB with molecular Trojan horse technology. A molecular Trojan horse is a peptide, or peptidomimetic MAb, that undergoes receptor-mediated transport across the BBB via an endogenous BBB receptor. The most potent molecular Trojan horse known is a HIRMAb, which enters brain via the endogenous BBB insulin receptor. The HIRMAb is biologically active in humans and Old World primates, such as the Rhesus monkey, but is not active in New World primates, or in rodents, or other lower species.

The engineering of a fusion protein of the HIRMAb and PON-1 represents a re-engineering of the PON1 protein to enable brain penetration and transport across the BBB. In the first generation fusion protein, AGT-185, the full PON-1 amino acid sequence, from Met-1 to Leu-355, was fused to the carboxyl terminus of the heavy chain of the HIRMAb. This sequence includes the PON1 signal peptide, from Met-1 to Ala-15, since the PON1 signal peptide is not cleaved in vivo. In the second generation fusion protein, AGT-186, a PON1 variant is fused to the HIRMAb, whereby the 15 amino acid signal peptide is deleted, and the His-115 is mutated to Trp-115, the H115W mutation.

Body

Original Statement of Work (SOW):

<u>01 year (months 1-12):</u>

(1) AGT-185

- (a) Dual transfection of COS cells with pCD-HC185 and pCD-LC1 with Lipofectamine 2000 in COS cells under SFM conditions (10xT500 flasks)
- (b) Collection of 2L of conditioned SFM and protein A affinity chromatography of AGT-185 in the presence of 1 mM calcium
- (c) Western blotting of purified AGT-185 with primary antibodies to human IgG and human PON-1
- (d) HIR binding assay using purified HIR extracellular domain as the receptor, and comparison of affinity of AGT-1 and AGT-185 (lot to be shipped to USAMRICD)

(e) Shipment of at least 25 ug of purified AGT-185 to USAMRICD for determination of activity against chemical nerve gas agents, and comparison with recombinant BCE or PON mutants (BCE or PON mutants provided by USAMRICD)

(2) AGT-186
(a) receipt from USAMRICD of a single plasmid DNA encoding mutated BCE or PON
(b) PCR of BCE or PON mutant cDNA and subcloning into pCD-UTV1 to produce pCD-HC186
(c) Bi-directional DNA sequencing of pCD-HC186 expression cassette, which includes CMV promoter, AGT-186 heavy chain fusion gene, and BGH transcription termination sequence
(d) Dual transfection of COS cells with pCD-HC186 and pCD-LC1
(e) Protein A affinity purification of AGT-186 from COS cell conditioned SFM
(f) Western blotting with primary antibodies to human IgG and human BCE/PON (BCE/PON antibody provided by USAMRICD if commercial antibody not satisfactory)
(g) HIR binding assay for comparison of binding of AGT-1, AGT-185, and AGT-186

02 year (months 13-24):

(1) AGT-185
(a) Dual transfection of COS cells with pCD-HC185 and pCD-LC1
(b) Collection of 2L of conditioned SFM and protein A affinity chromatography of AGT-185 in the presence of 1 mM calcium
(c) Radio-iodination of AGT-185 and purification
(d) Intravenous injection of [^{125}I]-AGT-185 in anesthetized adult Rhesus monkey, and measurement of plasma pharmacokinetics, brain uptake, peripheral organ uptake, and brain scanning

(2) AGT-186
(a) Dual transfection of COS cells with pCD-HC186 and pCD-LC1 with Lipofectamine 2000 in COS cells under SFM conditions (10xT500 flasks)
(b) Collection of 2L of conditioned SFM and protein A affinity chromatography of AGT-186
(c) Western blotting of purified AGT-186 with primary antibodies to human IgG and either human PON-1 or human BCE
(d) HIR binding assay using purified HIR extracellular domain as the receptor; comparison of affinity of AGT-1, AGT-185, and AGT-186
(e) Shipment of at least 25 ug of purified AGT-186 to USAMRICD for determination of activity against chemical nerve gas agents, and comparison with recombinant BCE or PON mutants (BCE or PON mutants provided by USAMRICD)

Key Research Accomplishments

- COS cells were dual transfected with the heavy chain (HC) and the light chain (LC) expression plasmids encoding AGT-185. Conditioned serum free medium (>2L) was collected, and AGT-185 was purified by protein A affinity chromatography in the presence of 1 mM CaCl2. The bi-functionality of the purified AGT-185 was verified by an HIR binding ELISA, a fluorometric enzymatic

assay of PON-1 organophosphatase activity, and by human IgG and PON1 Western blotting.

- The COS-derived AGT-185 was shipped to the USAMRICD, and shown to have activity against chemical nerve gas agents that was comparable to the PON1 control.
- After discussions with the USAMRICD, (Stephen Kirby), the novel formulation of AGT-186 was designed, and this genetic engineering program is now on-going. The Company will clone human PON1, minus the 15 amino acid signal peptide, by PCR from human liver cDNA, and will perform the H115W mutation by site-directed mutagenesis.

Reportable Outcomes

- No publications to date
- The data on the action of AGT-185 on chemical nerve gas agents was reported by Stephen Kirby at a closed USAMRICD meeting with poster presentation.

Conclusions

The results of the past year demonstrate that PON1 can be successfully re-engineered as a fusion protein with the HIRMAb. The organophosphatase enzyme activity of AGT-185 is comparable to PON1. However, PON1 does not cross the BBB, whereas AGT-185 is designed to cross the BBB in vivo. This will be verified in the upcoming year, following the radiolabeling of AGT-185, intravenous injection in an adult Rhesus monkey, and determination of the pharmacokinetics of plasma clearance, and the brain penetration of the fusion protein. We have indications that the fusion protein may be cleaved at the PON1 signal peptide junction. Since the amino terminus is not required for enzyme activity, the amino terminal signal peptide is removed in the engineering of AGT-186. The second generation AGT-186 will also include the H115W mutation identified by the USMRICD.

AGT-185 and AGT-186 are novel fusion proteins that represent a re-engineering of human PON-1 to enable BBB penetration by this enzyme, the most potent human organophosphatase. Nerve gas agents are small lipid soluble chemical compounds that rapidly cross the BBB and enter brain. Since nerve gas agents kill via a site of action within the CNS, behind the BBB, it is crucial that new nerve gas treatments penetrate the BBB, to degrade the organophosphates within the brain.

References

None.

Appendices

None.